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(54) Title: A METHOD OF DETECTING DRUG-RECEPTOR AND PROTEIN-PROTEIN INTERACTIONS

(57) Abstract

The present invention relates to methods for detecting interactions between two proteins as well as detecting the modulation of those interactions. The present invention is based upon the discovery of a new non-nuclear system utilizing G protein gamma subunit fusions to detect the interactions between two proteins and is particularly useful for the detection of the interaction between two or more proteins wherein one of the proteins is associated with the cell membrane. Related methods, compositions and kits can be used to detect or assay the interactions between essentially any two proteins that can be expressed in a cell.

A METHOD OF DETECTING DRUG-RECEPTOR AND PROTEIN-PROTEIN INTERACTIONS

Technical Field

The present invention relates to methods for detecting interactions between two

5   proteins as well as detecting the modulation of those interactions. The present invention is based upon the discovery of a new non-nuclear system to detect the interactions between two proteins and is particularly useful for the detection of the interaction between two or more proteins wherein one of the proteins is associated with the cell membrane. Related methods, compositions and kits can be used to detect or assay the

10   interactions between essentially any two proteins that can be expressed in a cell.

Acknowledgment of Federal Support

Background of the Invention

15   A fundamental area of inquiry in biology is the analysis of interactions between proteins. Proteins are complex macromolecules made up of covalently linked chains of amino acids. Each protein assumes a unique three dimensional shape determined principally by its sequence of amino acids. Many proteins consist of smaller units termed domains, which are continuous stretches of amino acids able to fold

20   independently from the rest of the protein. Some of the important forms of proteins are enzymes, polypeptide hormones, receptors, nutrient transporters, structural components of the cell, hemoglobins, antibodies, nucleoproteins, and components of viruses.

Protein-protein interactions enable two or more proteins to associate. A large number of non-covalent bonds form between the proteins when two protein surfaces are

25   precisely matched. These bonds account for the specificity of recognition. Protein-protein interactions are involved, for example, in the assembly of enzyme subunits; in antigen-antibody reactions; in forming the supramolecular structures of ribosomes, filaments, and viruses; in transport; and in the interaction of receptors on a

cell with growth factors and hormones. Products of oncogenes can give rise to neoplastic transformation through protein-protein interactions. For example, some oncogenes encode protein kinases whose enzymatic activity on cellular target proteins leads to the cancerous state. Other examples of protein-protein interaction include when a virus

5 infects a cell by recognizing a polypeptide receptor on the surface and when platelets aggregate during thrombosis.

Protein-protein interactions have been generally studied in the past using biochemical techniques such as cross-linking, co-immunoprecipitation and co-fractionation by chromatography. A disadvantage of these techniques is that

10 interacting proteins often exist in very low abundance and are, therefore, difficult to detect. Another major disadvantage is that these biochemical techniques involve only the proteins, not the genes encoding them. When an interaction is detected using biochemical methods, the newly identified protein often must be painstakingly isolated and then sequenced to enable the gene encoding it to be obtained. Another disadvantage

15 is that these methods do not immediately provide information about which domains of the interacting proteins are involved in the interaction.

To alleviate the problems associated with the biochemical characterization of protein-protein interactions, genetic systems have been invented that are capable of rapidly detecting which proteins interact with a known protein, determining which

20 domains of the proteins interact, and providing the genes for the newly identified interacting proteins. One such system is the yeast two-hybrid system wherein two proteins are expressed in yeast: one protein of interest fused to a DNA-binding domain and the other protein of interest fused to a transcriptional activation domain (Fields *et al.* (1989) *Nature* 340:245; Gyuris *et al.* (1993) *Cell* 75:791; Harper *et al.* (1993) *Cell*

25 75:805; Serrano *et al.* (1993) *Nature* 366:704; and Hannon *et al.* (1993) *Genes & Dev.* 7:2378). If the proteins interact, they activate transcription of a reporter gene that contains a binding site for the DNA-binding protein.

Although the development of genetic systems that utilize direct activation of a reporter gene, such as the yeast two-hybrid systems, has greatly facilitated the study of

30 protein-protein interactions, many problems remain to be solved. For instance, the yeast two-hybrid systems rely on interactions between the two proteins in the nucleus of the

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cell. Accordingly, yeast two-hybrid systems are not useful for the study of integral membrane protein interactions and cannot be used to test cell membrane impermeate drugs. Furthermore, the study of protein-protein interactions wherein one of the proteins is itself a transcriptional activator often results in the transcription of the reporter gene

5   without interaction between the two proteins under study. Lastly, the yeast two-hybrid systems require that both proteins under study be expressed as fusion proteins resulting in the possible loss of function.

Summary of the Invention

The present inventors have discovered a genetic system to study protein-protein

10   interactions that solves many of the problems associated with existing genetic systems. This system utilizes the receptor-G protein signaling system present in yeast and other eukaryotic cells to study protein-protein interactions, including the interactions wherein one protein is an integral membrane protein.

The present invention includes kits, vectors and methods for detecting one or

15   more interactions between two proteins, comprising the steps of providing a cell with a first protein fused to a G protein gamma subunit and a second protein; and determining whether the interaction of the G protein gamma subunit with an effector molecule has been modulated, thereby determining whether the first and second proteins interact.

The present invention also includes kits, vectors and methods to identify an agent

20   that modulates at least one interaction between two proteins, comprising the steps of exposing a cell to the agent, the cell comprising a first protein fused to a G protein gamma subunit and a second protein; and determining whether the interaction of the G protein gamma subunit with an effector molecule has been modulated.

The present invention further provides kits, vectors and methods to identify

25   binding partners of a protein, comprising the steps of transforming or transfecting host cells with a library comprising a population of nucleic acid molecules which express a first protein fused to a G protein gamma subunit to produce a host cell population, said population of nucleic acids differing with respect to the first protein fused to a G protein gamma subunit; transforming the host cell population with a vector which expresses a

30   second protein; culturing said host cell under conditions which express said first and

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second protein; and determining the activity of an effector molecule which is capable of interacting with the G protein gamma subunit.

Brief Description of the Drawings

5   **Figures 1A-1B.** Figures 1A and 1B are schematics comparing the traditional yeast two-hybrid methods of detecting protein-protein interactions to the methods of the invention. Proteins X and Y of Figure 1B correspond to the first and second proteins of the claimed methods, respectively.

       Figures 2A-2B. Figures 2A and 2B are schematics of pRS314-GAL-rbSec1-H6-STE18 and pRS316-ADH-syntaxin.

10   **Figures 3A-3B.** Figure 3A represents the growth inhibition results of an assay to detect the ability of Sec-1 and syntaxin to interact and prevent pheromone induced growth inhibition. Figure 3B is a schematic of the elements used in each growth inhibition assay.

Modes of Carrying Out the Invention

15   General Description

       The present invention utilizes the heterotrimeric G protein (guanine nucleotide binding regulatory protein) regulatory mechanisms found in all eukaryotic cells to study protein-protein interactions. When functioning normally, G proteins act as an integral part of the signal transduction mechanisms by which extracellular hormones and other

20 extracellular signaling molecules convey their signals through the plasma membrane of the cell and thus elicit appropriate intracellular responses.

       Heterotrimeric G proteins are composed of three polypeptide subunits, namely G alpha, G beta and G gamma. The conformation the alpha subunit and the degree of association between all three subunits changes during the signal transduction process.

25 These changes are associated with the hydrolysis of the nucleotide GTP to form GDP

5

and Pi. The binding sites for GTP and GDP as well as the GTPase catalytic site reside in the alpha subunit.

The G protein cycle which occurs each time a signal is conveyed across the membrane can be summarized as follows. In an unstimulated cell, the G proteins are

5   found in the resting state in which alpha, beta and gamma are complexed together and GDP is bound to G alpha. The binding of an appropriate hormone or other signaling molecule to a G protein coupled receptor at the cell surface initiates a signaling event when the ligand-activated receptor stimulates the alpha subunit to exchange GDP for GTP. In the active form, the alpha subunit, bound to GTP, dissociates from the beta-

10   gamma complex, and the subunits then interact specifically with cellular effector molecules to evoke a cellular response. G protein beta-gamma complexes, and in some instance alpha subunits, can interact with different effector systems (e.g., phospholipase C, adenylyl cyclase systems, etc.) to evoke a variety of cellular responses.

The present invention uses the ability of the dissociated beta-gamma complex to

15   specifically interact with cellular effector molecules to evoke a cellular response as an endpoint in assays to detect protein-protein interactions. This ability of the beta-gamma complex to interact with cellular effector molecules generally requires that the beta-gamma complex be free of the alpha subunit. The present inventors have discovered that interactions between the beta-gamma complex dissociated from the alpha subunit and an

20   effector molecule can be prevented or inhibited by the association of the beta-gamma complex with another unrelated protein.

The methods of the invention comprise the expression of a first protein of interest fused in frame to a G protein gamma subunit. This G protein gamma subunit fusion is then expressed in the same cell as a second test protein which is typically expressed in

25   native form. Interactions, such as binding, between the first protein fused to a G protein gamma subunit and the second test protein are assayed by determining whether the fused G protein gamma subunit in its association with the beta subunit is capable of interacting with effector molecules. In instances where the first protein fused to a G protein gamma subunit and the second protein interact or bind, the fused G protein gamma subunit in its

30   association with the beta subunit is not free to interact with effector molecules. In contrast, when the first protein fused to a G protein gamma subunit and the second

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protein do not interact or bind, the fused G protein gamma subunit in its association with the beta subunit is free to interact with effector molecules (see Figure 1).

Specific Embodiments

Definitions

5   As used herein, the term "agent" means any molecule that is randomly selected or rationally designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the proteins under study or the known functions of the proteins under study. Examples of randomly selected agents are chemical libraries, peptide

10   combinatorial libraries, or a growth broth of an organism.

As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the proteins under study and/or their conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the amino acid

15   sequences that make up potential contact sites between the proteins. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to an identified contact site on one of the proteins under study. Such an agent will reduce or block the association of the protein with its binding partner by binding to the contact site on the first protein.

20   The agents of the present invention can be, as examples, peptides, small molecules, nucleic acids, vitamin derivatives, as well as carbohydrates. A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention.

The peptide agents of the invention can be prepared using standard solid phase

25   (or solution phase) peptide synthesis methods, as is known in the art. In addition, the DNA encoding these peptides may be synthesized using commercially available oligonucleotide synthesis instrumentation and produced recombinantly using standard recombinant production systems. The production using solid phase peptide synthesis is necessitated if non-gene-encoded amino acids are to be included.

Another class of agents are antibodies immunoreactive with one of the proteins under study. Particularly useful are antibodies immunoreactive with the extracellular domain of membrane proteins under study. As described above, antibodies are obtained by immunization of suitable mammalian subjects with peptides, containing as antigenic

5   regions, those portions of the protein intended to be targeted by the antibodies. Critical regions include the contact sites between the two proteins as well as extracellular regions of membrane proteins.

As used herein, the phrase "at least one interaction" refers to an interaction between the first protein fused to a G protein gamma subunit and the second protein

10   detectable in the methods of the invention. Such interactions are typically, but not always, a form of direct interaction such as binding.

As used herein, the term "modulates" refers to any change in an interaction between the first protein fused to a G protein gamma subunit and the second protein, wherein that change is detectable by the methods of the invention. The term "modulates"

15   includes inhibition or promotion of at least one interaction between a first protein fused to a G protein gamma subunit and the second protein.

As used herein, the term "cell" refers to any eukaryotic cell which contains a G protein coupled signaling system. Preferred cells include, but are not limited to, yeast cells such as *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* as well as insect,

20   plant and mammalian cells in culture.

As used herein, the term "fused" refers to a protein which has been expressed from a recombinant gene which comprises the coding sequence from the first protein covalently attached in frame to a G protein gamma subunit gene. This recombinant gene when transcribed and translated produces a chimeric protein comprising the amino acid

25   sequence of the first protein and the amino acid sequence of a G protein gamma subunit. Typically, the first gene is fused in frame to the coding sequence of a G protein gamma subunit to produce a chimeric protein wherein the first protein is fused to the N or amino terminus of the G protein gamma subunit.

As used herein, the phrase "G protein gamma subunit" refers to any available G

30   protein gamma subunit from a eukaryotic cell. G protein gamma subunits are known to be highly variable with over eleven gamma subunit genes isolated and various splice

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variants identified. See Hildebrand (1997) *Biochem. Pharm.* 54:325-339. The phrase

also includes fragments of a G protein gamma subunit that retain the ability to interact

with a G protein beta subunit and retain the ability to interact with at least one effector

molecule. Preferably, G protein gamma subunits are selected from species

5   corresponding to the selected host cell.

The term "first protein" refers to any protein employed in the methods of the

invention to detect protein-protein interactions. The first test protein is fused to a G

protein gamma subunit.

The term "second protein" refers to any protein employed in the methods of the

10   invention to detect protein-protein interactions. The second test protein is not limited to

any specific class of cellular proteins but specifically includes membrane proteins.

As used herein, the term "effector molecule" refers to any G protein gamma-beta

target that results in an assayable phenotype upon interaction with a G protein gamma-

beta complex. Many G protein effector molecules or targets have been identified

15   including ion channels I_{KG}, and I_{Ca}, phospholipase A_2, *STE20*, *STE5*, PLCβ, adenylyl

cyclases, G protein coupled receptor kinases, PI3K, the MAP kinase cascade, Bruton

tyrosine kinase, Tsk tyrosine kinase, arrestins and phosphodiesterases. See Clapham *et*

al. ((1997) *Ann. Rev. Pharmacol. Toxicol* 37:167-203) for a review of known effector

molecules or targets.

20   As used herein, the term "membrane protein" refers to a protein that comprises at

least one membrane spanning domain. Such membrane spanning domains typically

comprise as series of hydrophobic amino acid residues that span the lipid bilayer

membrane of a cell.

As used herein, the phrase "native form" refers to the expression of a protein

25   without alteration of its primary amino acid sequence and/or secondary structure. For

instance, a protein expressed in its native form is not expressed as a fusion protein.

As used herein, the term "sequestered" refers to any means of preventing the

interaction of a gamma-beta complex which is dissociated from the alpha subunit with an

effector molecule. The term includes, but is not limited to, the physical retention of the

30   gamma-beta complex in or on the cell membrane by the interaction of the first and

second proteins.

As used herein, the term "library" generally refers to either a collection of cDNA molecules corresponding to a population of mRNA isolated from a selected cell type, or the proteins encoded by the cDNA library. Methods to produce cDNA libraries from a selected cell population are commonly available, such as the methods disclosed by

5   Sambrook *et al.* (Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989). cDNA libraries from selected cell types are also commercially available from vendors such as Stratagene®.

As used herein, the term "detectable phenotype" refers to any phenotype which may be detected by physical, visual or chemical means elicited by the interaction of a

10 beta-gamma complex with an effector molecule. Detectable phenotypes utilized in the invention include but are not limited to yeast pheromone induced growth inhibition, other forms of detectable cellular morphology changes as well as the activities of reporter genes such as *lacZ* and *HIS3* operably linked to pheromone responsive promoters such as the *BAR*1 or *FUS*1 promoters (see U.S. Patent 5,482,835).

15

Methods for detecting protein-protein interactions

In one embodiment, the present invention makes use of a gene hybrid that encodes a first protein fused to a G protein gamma subunit. Interactions, such as binding, between the first protein fused to a G protein gamma subunit and the second test

20 protein is assayed by determining whether the fused G protein gamma subunit in its association with the beta subunit is capable of interacting with effector molecules upon induction of a G protein signaling cascade. In instances where the first protein fused to a G protein gamma subunit and the second test protein interact or bind, the fused G protein gamma subunit in its association with the beta subunit is not free to interact with effector

25 molecules. In contrast, when the first protein fused to a G protein gamma subunit and the second protein do not interact or bind, the fused G protein gamma subunit in its association with the beta subunit is free to interact with effector molecules (see Figure 1).

The methods of the invention include providing a host cell, preferably a yeast cell, most preferably *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*. In one

format, a first chimeric gene is provided. The first chimeric gene comprises a DNA sequence that encodes a first protein or fragment fused to a G protein gamma subunit.

A second gene is provided which is capable of being expressed in the host cell. The second gene encodes a second test protein which will typically be expressed in its native form. In one embodiment, both the first and the second genes are introduced into the host cell cloned into appropriate plasmids or expression vectors. The first chimeric gene may also be present in a chromosome of the host cell and the second gene is introduced into the host cell as part of a vector, or vice-versa. In another embodiment, the second gene may be natively expressed in the host cell.

Vectors useful for practicing the present invention include plasmids, viruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by homologous or nonhomologous recombination). The vector may replicate and function independently of the host genome, as in the case of a plasmid, or may integrate into the genome itself, as in the case of an integratable DNA fragment. Suitable vectors will contain replicon and control sequences which are derived from species compatible with the intended expression host. For example, a promoter operable in a host cell is one which binds the RNA polymerase of that cell, and a ribosomal binding site operable in a host cell is one which binds the endogenous ribosomes of that cell.

Transformation or transfection of the vectors into suitable host cells may be accomplished by any means available, such as those disclosed by Ausubel *et al.* ((1987), Current Protocols in Molecular Biology, Wiley-Interscience, New York). Transformed host cells employed in the present invention are cells which have been transformed or transfected with the vectors constructed using recombinant DNA techniques and express the protein or protein subunit coded for by the heterologous DNA sequences. The host cells may be, but are not required to be, incapable of expressing an endogenous G protein gamma-subunit corresponding to the G protein gamma subunit to which the first protein is fused.

Although the methods of the present invention are not limited to yeast cells, a variety of yeast cultures, and suitable expression vectors for transforming yeast cells, are known. See, *e.g.*, U.S. Pat. No. 4,745,057; U.S. Pat. No. 4,797,359; U.S. Pat. No. 4,615,974; U.S. Pat. No. 4,880,734; U.S. Pat. No. 4,711,844; and U.S. Pat. No.

4,865,989. *Saccharomyces cerevisiae* is the most commonly used among the yeast,
although a number of other strains are commonly available. See, *e.g.*, U.S. Pat. No.
4,806,472 (*Kluveromyces lactis* and expression vectors therefor); and U.S. Pat. No.
4,855,231 (*Pichia pastoris* and expression vectors therefor).

5   Yeast vectors may contain an origin of replication from the 2 micron yeast
plasmid or an autonomously replicating sequence (ARS), a promoter, DNA encoding the
heterologous DNA sequences, sequences for polyadenylation and transcription
termination, and a selection gene. An exemplary plasmid is YRp7, (Stinchcomb *et al.*,
(1979) *Nature* 282:39; Kingsman *et al.*, (1979) *Gene* 7:141; or Tschemper *et al.*, (1980)

10   *Gene* 10:157). This plasmid contains the TRP1 gene, which provides a selection marker
for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC
No. 44076 or PEP4-1 (Jones, (1977) *Genetics* 85, 12). The presence of the *trp1* lesion in
the yeast host cell genome then provides an effective environment for detecting
transformation by growth in the absence of tryptophan.

15   Suitable promoting sequences in yeast vectors include the promoters for
metallothionein, 3-phosphoglycerate kinase (Hitzeman *et al.*, (1980) *J. Biol. Chem.* 255:
2073 or other glycolytic enzymes (Hess *et al.*, (1968) *J. Adv. Enzyme Reg.* 7:149; and
Holland *et al.*, (1978) *Biochemistry* 17:4900), such as enolase,
glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase,

20   phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase,
pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.
Suitable vectors and promoters for use in yeast expression are further described in R.
Hitzeman *et al.*, EPO Publn. No. 73,657. Other promoters, which have the additional
advantage of transcription controlled by growth conditions, are the promoter regions for

25   alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes
associated with nitrogen metabolism, and the aforementioned metallothionein and
glyceraldehyde-3-phosphate dehydrogenase, as well as enzymes responsible for maltose
and galactose utilization.

In constructing suitable expression plasmids, the termination sequences

30   associated with these genes may also be ligated into the expression vector 3' of the

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heterologous coding sequences to provide polyadenylation and termination of the
mRNA.

As discussed above, induction of a G protein signaling cascade may be required
to induce a detectable phenotype which is dependent upon the interaction of the first and
5   second proteins used in the invention. The choice of inducing agent depends in part
upon the choice of detectable phenotype selected as well as the effector molecule
involved in the signaling pathway. For instance, in yeast wherein pheromone induced
growth inhibition is used as the detectable phenotype, α-factor pheromone is employed
to induce the G protein signaling cascade. Alternatively, available agonists of any G
10   protein-coupled receptor may be used to induce a specific phenotype such as hormones,
neurotransmitters and growth factors (see Muller *et al.* (1995) *Biochem. Soc. Trans.*
23(1):141-8 and Dohlman *et al.* (1997) *J. Biol. Chem.* 272(7):3871-3874). Exposure to
differing amounts of an inducing agent may be used to modulate the detectable
phenotype.

15   The above methods may be modified to identify agents which modulate at least
one interaction between the first and second proteins. In this format, vectors encoding a
first protein fused to a G protein gamma subunit and a second protein are prepared.
Generally, the first and second protein will be previously identified. Further, the
interaction between the first protein and the second protein will typically be associated
20   with a given disease state, condition or clinical indication.

Cells which express a first protein fused to a G protein gamma subunit and a
second protein are exposed or incubated with the agent. The cells are then assayed for
the ability of the agent to modulate an interaction between the first and second protein.
In this format, cells exposed to the agent which inhibits an interaction between the first
25   and second proteins will exhibit changes in the detectable phenotype being assayed. This
change in detectable phenotype occurs, after induction of a G protein signaling cascade,
when the G protein subunits dissociate and G beta/gamma is free to activate a signaling
pathway leading to the detectable phenotype. In control cells not exposed to the agent,
the interaction of the first and second proteins prevents beta/gamma from interacting
30   with the normal effector to activate the signaling pathway.

The above methods may be used to identify new, previously unidentified binding partners for a known protein. In one format, a known protein may be expressed in its native form as the second protein to screen for potential binding partners expressed from a library of G protein gamma subunit fusions as the first protein. In another format, the

5   first protein may be a known protein expressed as a G protein gamma subunit fusion to screen for potential binding partners expressed as the second protein from a cDNA library.

The methods of the invention may also be modified to detect and identify mutations which modulate protein-protein interactions between two binding partners. As

10   an example, mutations in a first protein fused to a G protein gamma subunit which affect the binding to a second protein may be assayed by preparing a library of mutations fused in frame to the G protein gamma subunit. Mutations may be produced by any means available in the art, such as site directed (oligonucleotide-directed) mutagenesis, linker insertion or scanning mutagenesis, deletion mutagenesis or mutations induced in Sec-1

15   by chemical mutagenesis. See Sambrook et al. for a variety of available site-directed and random mutagenesis methods.

In an alternative format, mutations in a second protein which affect the binding of the second protein to a first protein fused to a G protein gamma subunit may be assayed by preparing a library of mutations in the second protein. Mutations in the

20   second protein may also be prepared using any available means including site directed (oligonucleotide-directed) mutagenesis, linker insertion or scanning mutagenesis, deletion mutagenesis or mutations induced by chemical mutagenesis.

The methods of the present invention, as described above, may be practiced using a kit for detecting interactions between a first test protein and a second test protein. The

25   kit may include a container, two vectors, and a host cell. The first vector contains a promoter and may include a transcription termination signal functionally associated with the coding sequence for a G protein gamma subunit. The first vector includes a restriction site(s) for inserting a DNA sequence encoding a first test protein or protein fragment in such a manner that the first test protein is expressed as part of a fusion

30   protein with the G protein gamma subunit. The first vector may include the coding sequence for a known protein fused in frame to the coding sequence for a G protein

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gamma subunit. Alternatively, the first vector may be supplied as a population of vectors comprising a library of cDNA fragments fused in frame to the coding sequence for a G protein gamma subunit.

The kit includes a second vector which includes a promoter and a transcription
5   termination signal to direct transcription. The second vector also includes a restriction site(s) to insert a DNA sequence encoding the second test protein or protein fragment into the vector, in such a manner that the second test protein is capable of being expressed in a relevant host cell. The second vector may include the coding sequence for a known protein. Alternatively, the second vector may be supplied as a population of
10   vectors comprising a library of cDNA fragments.

The kit may also include a host cell, preferably a yeast strain of *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe*.

The following working examples specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of
15   the disclosure. Other generic configurations will be apparent to one skilled in the art.

Example 1   Production of G Protein Gamma Subunit Fusion Vectors.

The production of G protein gamma fusions to a protein of interest may be accomplished using any available vectors designed to replicate and express the fusion protein in the appropriate host cell.
20   When yeast cells are used as the host cell, pRS316-*GAL-STE*4 was constructed by ligation of the *Eco*RI-*Sal*I fragment from pL19, containing the bidirectional galactose-inducible *GAL*1/10 promoter and *STE*4 (Gβ gene) (Whiteway *et al.*, (1990) Overexpression of the *STE4* gene leads to mating response in haploid *Saccharomyces cerevisiae, Molecular and Cellular Biology* 10:217-222.) into the vector pRS316
25   (described by Sikorski *et al.*, (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics* 122:19-27.). *STE*18 (Gγ gene) was PCR amplified using the plasmid M57p4 as the template (Whiteway *et al.*) and oligonucleotides that flank the *STE*18 open reading frame (GGG AAT TCA ATG GCT CAC CAT CAC CAT CAC CAT GCT AGC ATG ACA TCA GTT CAA AAC
30   TCT CC, (SEQ ID NO: 1) 5' oligo, encoding 6 His residues upstream of the amino

terminus; GAG GCT CTA CGT AGC AAG, (SEQ ID NO: 2) 3'oligo). The PCR product was digested with *Eco*RI and *Sac*I, and ligated to the corresponding sites in pRS316-*GAL-STE*4 described above to produce pRS316-*GAL-STE*4/18-H6.

pRS314-*GAL*-H6-*STE*18 (Figure 2) was constructed by ligation of the *Bam*HI-
5   *Sac*I fragment from pRS316-*GAL-STE*4/18-H6 into pRS314. To prepare fusions of a protein of interest to the yeast G protein gamma subunit, the coding sequence of the protein is inserted in frame into the *Eco*RI site of pRS314-*GAL*-H6-*STE*18 to yield pRS314-GAL-heterologous gene-H6-*STE*18.

10   *Example 2*   A Method of Detecting the Interaction Between Two Proteins
        *Production of a Rat Sec-1- G Protein Gamma Subunit Fusion and
Transformation of Host Cells Expressing the Sec-1 G Protein Gamma Subunit Fusion
and Syntaxin.*
        Strains, Media and Transformation:
15   Standard methods for the growth, maintenance, and transformation of yeast and bacteria, and for the manipulation of DNA, were used throughout (Ausubel *et al.* 1987, Current Protocols in Molecular Biology. Wiley-Interscience, New York). The Escherichia coli strain DH5α was used for the maintenance of plasmids. Yeast cells were grown in synthetic medium supplemented with amino acids, adenine and 2% glucose (SCD) or 2%
20   galactose plus 0.2% sucrose (SCG); tryptophan and/or uracil were omitted to maintain selection for plasmids. The *Saccharomyces cerevisiae* strain used in this study was MHY6 (from Jeremy Thorner) (MATa *ura3*-52 *lys2*-801[am] *ade2*-101[oc] *trp*1-Δ63 *his3*-Δ200 *leu2*-Δ1 *ste*18Δ::*LEU2*) (derived from Sikorski *et al.* (1989) *Genetics* 122(1):19-27).
25

        Plasmid Construction:
        pRS316-*GAL-STE*4 was constructed by ligation of the *Eco*RI-*Sal*I fragment from pL19, containing the bidirectional galactose-inducible *GAL*1/10 promoter and *STE*4 (Gβ gene) (from Whiteway *et al.*) into the vector pRS316 (described in Sikorski *et al.*, (1989)
30   A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae. Genetics* 122:19-27.). *STE*18 (Gγ gene) was PCR

amplified using the plasmid M57p4 as the template (from Whiteway *et al.*) and

oligonucleotides that flank the *STE*18 open reading frame (GGG AAT TCA ATG GCT CAC

CAT CAC CAT CAC CAT GCT AGC ATG ACA TCA GTT CAA AAC TCT CC, (SEQ ID NO: 1) 5'

oligo, encoding 6 His residues upstream of the amino terminus; GAG GCT CTA CGT AGC

5   AAG, (SEQ ID NO: 2) 3'oligo). The PCR product was digested with *Eco*RI and *Sac*I, and

ligated to the corresponding sites in pRS316-*GAL-STE*4 described above to produce

pRS316-*GAL-STE*4/18-H6.

        pRS314-*GAL*4-H6-*STE*18 was constructed by ligation of the *Bam*HI-*Sac*I

fragment from pRS316-*GAL-STE*4/18-H6 into pRS314. Rat brain Sec1 (rbSec1) was

10   PCR amplified using a plasmid that contains the rbSEC cDNA in pBluescript II SK as a

template (Garcia *et al.*, (1994) A rat brain Sec1 homologue related to Rop and UNC18

interacts with syntaxin. *Proc. Nat'l Acad. Sci.* USA 91:2003-7) and oligonucleotides that

flank the Sec1 open reading frame (CCG AAT TCC ATG GCC ATT GGC CTC AAG,

(SEQ ID NO:3) 5' oligo, containing an *Eco*RI site beginning 7 nucleotides upstream of

15   the initial ATG; CCT GAA TTC GAG CTT ATT TCT TCG TCT GTT TTA TTC AG,

(SEQ ID NO: 4) 3' oligo, containing an *Eco*RI site in frame with the N-terminal open

reading frame of His6-*STE*18). The rbSec1 PCR product was digested with *Eco*RI and

ligated into the *Eco*RI site of pRS314-*GAL*-H6-*STE*18 to yield pRS314-*GAL*-rbSec1-H6-

*STE*18.

20   pRS316-ADH (amp^r, CEN/ARS, *URA*3 and *ADH*1 promoter and termination

sequence) (described in Song *et al.*, (1996) Regulation of membrane and subunit

interactions by N-myristoylation of a G protein α subunit in yeast. *J. Biol. Chem.*

271:20273-20283.). Rat syntaxin 1a was PCR amplified using a cDNA in vector CDM8

as the template, (Blasi *et al.* (1993). Botulinum neurotoxin C1 blocks neurotransmitter

25   release by means of cleaving HPC-1/syntaxin. *EMBO J.* 12:4821-8.) and

oligonucleotides that flank the syntaxin open reading frame (CGC GGG TCG ACA TGA

AGG ACC GAA CCC AGG, (SEQ ID NO:5) 5' oligo, containing a *Sal*I site beginning 6

nucleotides upstream of the initial ATG; CCG AAT TCA CTA TCC AAA GAT GCC

CCC GAT GG, (SEQ ID NO: 6) 3' oligo, containing an *Eco*RI site 10 nucleotides

30   downstream of the termination codon). The PCR amplified product was digested with

*Sal*I and *Eco*R1 and ligated to the corresponding sites in pRS316-*ADH*.

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Detection of Sec-1-Syntaxin Interactions

Growth Inhibition Assay:

To evaluate to the interactions of the Sec-1 G protein gamma subunit fusion and
syntaxin, a growth inhibition assay (halo assay) was carried out by diluting 50 µL from
5   an overnight culture grown at 30°C in selective media with 2 mL sterile water, followed
by the addition of an equal volume of 1% (w/v) dissolved agar (55°C) and poured onto a
culture dish of SCD-Trp-Ura or SCG-Trp-Ura medium. Sterile filter discs were spotted
with 5 µg or 15 µg of synthetic α-factor pheromone and placed onto the nascent lawn to
induce growth arrest. The resulting zone of growth-arrested cells was documented after
10   2 days at 30°C.

As set forth in Figure 3, yeast expressing the Sec-1 G protein gamma subunit
fusion and syntaxin did not respond to α-factor induced growth arrest as exhibited by no
zone of inhibition around filter paper disks containing 5 or 15 µg of α-factor. This is in
contrast to control cells which express the Sec-1 G protein gamma fusion alone and cells
15   which express syntaxin alone. In the control cells, upon pheromone addition, the G
protein subunits dissociate and G beta/gamma is free to activate a signaling pathway
leading to growth arrest. When the cells express the Sec-1 G protein gamma fusion and
syntaxin, pheromone induced growth arrest is blocked because the interaction of syntaxin
with Sec1 prevents the beta/gamma from interacting with the normal effector to activate
20   the signaling pathway to growth arrest.

Example 3   Methods for Testing the Ability of an Agent to Modulate an Interaction
Between Two Proteins.

The system described in Example 2 may be used to screen for agents which
modulate the interactions between any two proteins. As an example, agents which
25   modulate the interactions between Sec-1 and syntaxin may be screened by exposing
yeast cells expressing the Sec-1 G protein gamma fusion and syntaxin to the agent to be
tested. A growth inhibition assay (halo assay) is carried out by diluting 50 µL from an
overnight culture of yeast expressing the Sec-1 G protein gamma fusion and syntaxin at
30°C in selective media with 2 mL sterile water, followed by the addition of an equal
30   volume of 1% (w/v) dissolved agar (55°C) and poured onto a culture dish of SCD-Trp-

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Ura or SCG-Trp-Ura medium with and without the agent to be tested. Sterile filter discs are spotted with 5 μg or 15 μg of synthetic α-factor pheromone and placed onto the nascent lawn to induce growth arrest. Alternatively, the agent to be tested may be spotted onto filter disks with the synthetic α-factor pheromone. The resulting zone of

5   growth-arrested cells is then documented after 2 days at 30°C.

In control cells not exposed to the agent, pheromone induced growth arrest is blocked because the interaction of syntaxin with Sec1 prevents beta/gamma from interacting with the normal effector to activate the signaling pathway to growth arrest. In cells exposed to an agent which inhibits the interaction of Sec-1 and syntaxin, upon

10   pheromone addition, the G protein subunits dissociate and G beta/gamma is free to activate a signaling pathway leading to growth arrest. This pheromone induced growth arrest is exhibited by a zone of inhibition around the filter disk.

In a second assay, agents which modulate the interactions between the Her-2/neu receptor tyrosine kinase which is upregulated in some cancerous cells, including some

15   breast cancers, and Grb2 may be screened by exposing yeast cells expressing a Grb2-G protein gamma fusion and Her-2/neu to the agent to be tested. A vector comprising a Grb2-G protein gamma fusion is prepared by inserting the coding sequence for Grb2 (available in Lowenstein *et al.* (1992) Cell 70(3):431-442) into pRS314-*GAL*4-H6-*STE*18. A second vector encoding Her-2/neu is prepared by insertion of the coding

20   sequence of Her-2/neu (available in Yamamoto *et al* (1986) *Nature* 319:230-234) into pRS316-ADH.

A growth inhibition assay (halo assay) is carried out by diluting 50 μL from an overnight culture of yeast expressing the Grb2-G protein gamma fusion and Her-2/neu at 30°C in selective media with 2 mL sterile water, followed by the addition of an equal

25   volume of 1% (w/v) dissolved agar (55°C) and poured onto a culture dish of SCD-Trp-Ura or SCG-Trp-Ura medium with and without the agent to be tested. Sterile filter discs are spotted with 5 μg or 15 μg of synthetic α-factor pheromone and placed onto the nascent lawn to induce growth arrest. Alternatively, the agent to be tested may be spotted onto filter disks with the synthetic α-factor pheromone. The resulting zone of

30   growth-arrested cells is then documented after 2 days at 30°C.

In control cells not exposed to the agent, pheromone induced growth arrest is
blocked because the interaction of activated Her-2/neu with Grb2 prevents beta/gamma
from interacting with the normal effector to activate the signaling pathway to growth
arrest. In cells exposed to an agent which inhibits the interaction of activated Her-2/neu

5   and Grb2, upon pheromone addition, the G protein subunits dissociate and G
beta/gamma is free to activate a signaling pathway leading to growth arrest. This
pheromone induced growth arrest is exhibited by a zone of inhibition around the filter
disk.

Example 4   Method for Identifying Binding Partners for a Protein

10   The system described in Example 2 may also be used to identify new, previously
unidentified binding partners for a known protein. In one format, a known protein may
be expressed in its native form, such as syntaxin, to screen for potential binding partners
expressed from a library of G protein gamma subunit fusions. Syntaxin is a known target
for botulinum toxin. Botulinum toxin is currently used as a therapeutic agent to treat

15   central nervous system disorders such as spasticity among stroke patients. The
identification of new binding partners for syntaxin may elucidate alternate potential drug
targets for the treatment of such disorders.

Rat syntaxin 1a is PCR amplified using a cDNA in vector CDM8 as the template,
(Blasi, J., E. R. Chapman, S. Yamasaki, T. Binz, H. Niemann, and R. Jahn, 1993.

20   Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-
1/syntaxin. EMBO Journal 12:4821-8) and oligonucleotides that flank the syntaxin open
reading frame (CGC GGG TCG ACA TGA AGG ACC GAA CCC AGG, (SEQ ID NO:
5) 5' oligo, containing a *Sal*I site beginning 6 nucleotides upstream of the initial ATG;
CCG AAT TCA CTA TCC AAA GAT GCC CCC GAT GG, (SEQ ID NO: 6) 3' oligo,

25   containing an *Eco*RI site 10 nucleotides downstream of the termination codon). The
PCR amplified product was digested with *Sal*I and *Eco*RI and ligated to the
corresponding sites in pRS316-*ADH* to facilitate expression of rat syntaxin 1a in a
recombinant yeast cell.

First Protein Library Fusions to G Protein gamma Subunit

20

A library of G Protein gamma subunit fusions is prepared by ligating a cDNA library of choice into the *Eco*RI site of pRS314-*GAL*-H6-*STE*18 as described above. Alternatively, appropriate vector backbones may be substituted depending on the cell in which the G protein gamma subunit fusions are to be expressed.

5   To prepare the library of G protein gamma subunit fusions, the cDNA inserts from a rat brain cDNA library purchased from Stratagene (Cat. No. 936501) are released from the backbone vector using standard procedures and inserted into the *Eco*RI site of pRS314-*GAL*-H6-*STE*18. This library is then transformed into yeast which express rat syntaxin 1a and a *lacZ* reporter gene fused downstream from the *FUS*1 pheromone

10   inducible promoter (Nomoto *et al.* (1990) EMBO J. 9:691) by the methods of Ausubel *et al.*

Screening and identification for proteins which interact with syntaxin are accomplished by assaying for the ability of individual fusion proteins from the G protein gamma subunit fusion library to interact with syntaxin after pheromone induction. In

15   cells which express a G protein gamma subunit fusion which does not interact with syntaxin, upon pheromone exposure, the G protein subunits dissociate and G beta/gamma is free to activate a signaling pathway leading to transcription of the *lacZ* reporter gene fused downstream from the *FUS*1 promoter. In cells which express a G protein gamma fusion which interacts with syntaxin, pheromone induced transcription

20   from the *lacZ* reporter gene fused downstream from the *FUS*1 promoter is blocked. In this case, the interaction of the G protein gamma subunit fusion with syntaxin prevents beta-gamma from activating a signaling pathway leading to transcription of the *lacZ* reporter gene fused downstream from the *FUS*1 promoter

Individual G protein gamma subunit fusions which may interact with syntaxin are

25   isolated by recovery and sequencing of the cDNA insert fused to the coding sequence for the G protein gamma subunit by standard cloning and sequencing techniques. Such techniques are widely available such as those disclosed by Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Plainview N.Y., 1989).

30   *Expression of cDNA Libraries as the Second Protein.*

In an alternative format, the rat brain library may be screened for proteins which interact with the Sec-1/ G protein gamma subunit fusion expressed by pRS314-*GAL*-rbSec1-H6-*STE*18. In this format, the rat brain library cDNA described above is released from the backbone vector using standard procedures and inserted into pRS316-ADH.

5   This library is then co-transformed into cells which contain a *lacZ* reporter gene fused downstream from the *FUS*1 pheromone inducible promoter (Nomoto *et al.* (1990) EMBO J. 9:691) with pRS314-*GAL*-rbSec1-H6-*STE*18.

Proteins which interact with Sec-1 are identified by assaying for the ability of individual proteins encoded by the library to interact with Sec-1 after pheromone

10   induction. In cells which express a protein encoded by a member of the rat cDNA library which does not interact with the Sec-1/G protein gamma subunit fusion, upon pheromone exposure, the G protein subunits dissociate and G beta-gamma is free to activate a signaling pathway leading to transcription of the *lacZ* reporter gene fused downstream from the *FUS*1 promoter. In cells which express a library protein which does interact

15   with the Sec-1/G protein gamma fusion, pheromone induced transcription from the *lacZ* reporter gene fused downstream from the *FUS*1 pheromone inducible promoter is blocked. In this case, the interaction of the Sec-1/G protein gamma subunit fusion with the library protein prevents the beta/gamma from activating a signaling pathway leading to transcription of the *lacZ* reporter gene fused downstream from the *FUS*1 promoter.

20   Individual cDNAs encoding library proteins which may interact with Sec-1 are isolated by recovery and sequencing of the cDNA insert by standard cloning and sequencing techniques. Such techniques are widely available such as those disclosed by Sambrook *et al.* (Molecular Cloning: A Laboratory Manual, 2nd edition, Cold SPRING Harbor Laboratory Press, Plainview N.Y., 1989).

25   *Example 5*   Method for Identifying Mutations which Disrupt Protein-Protein Interactions.

The system described in Example 2 may also be used to screen for the effects of mutations which modulate protein-protein interactions between two binding partners. As an example, mutations in Sec-1 which effect the binding of Sec-1 to syntaxin may be

30   assayed by preparing a library of Sec-1 mutations fused in frame to the G protein gamma

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subunit as described above. Mutations in Sec-1 may be produced by any means available in the art, such as site directed (oligonucleotide-directed) mutagenesis, linker insertion or scanning mutagenesis, deletion mutagenesis or mutations induced in Sec-1 by chemical mutagenesis. See Sambrook *et al.* for a variety of available site-directed and random

5   mutagenesis methods.

Mutations in Sec-1 are then screened for their ability to disrupt Sec-1 binding to syntaxin by transforming cells which express syntaxin from pRS316-ADH-syntaxin with pRS314-*GAL*-rbSec1(mut)-H6-*STE*18 (rbSec-1(mut) encoding a Sec-1 mutation). Growth inhibition assays (halo assays) are carried out by diluting 50 µL from an

10 overnight culture grown at 30°C in selective media with 2 mL sterile water, followed by the addition of an equal volume of 1% (w/v) dissolved agar (55°C) and poured onto a culture dish of SCD-Trp-Ura or SCG-Trp-Ura medium. Sterile filter discs are spotted with 5 µg or 15 µg of synthetic α-factor pheromone and placed onto the nascent lawn to induce growth arrest. The resulting zone of growth-arrested cells is documented after 2

15 days at 30°C.

Yeast expressing wild-type (non-mutated) Sec-1 G protein gamma subunit fusion and syntaxin do not respond to α-factor induced growth arrest as exhibited by no zone of inhibition around filter paper disks containing 5 or 15 µg of α-factor. This is contrast to cells which express Sec-1 G protein gamma fusions wherein Sec-1 contains a mutation

20 that disrupts Sec-1-syntaxin binding. In these cells, upon pheromone addition, the G protein subunits dissociate and G beta/gamma is free to activate a signaling pathway leading to growth arrest.

In an alternative format, mutations in syntaxin which affect the binding of syntaxin to Sec-1 may be assayed by preparing a library of syntaxin mutations as

25 described above. Mutations in syntaxin may be prepared by any available means including site directed (oligonucleotide-directed) mutagenesis, linker insertion or scanning mutagenesis, deletion mutagenesis or mutations induced in syntaxin by chemical mutagenesis.

Mutations in syntaxin are then screened for their ability to disrupt syntaxin

30 binding to Sec-1 by transforming cells which express a Sec-1/G protein gamma subunit fusion, such as that encoded by pRS314-*GAL*-rbSec1-H6-*STE*18 with pRS316 ADH-

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syntaxin* (wherein "syntaxin*" refers to a syntaxin mutation). Growth inhibition assays (halo assays) are carried out by diluting 50 μL from an overnight culture grown at 30°C in selective media with 2 mL sterile water, followed by the addition of an equal volume of 1% (w/v) dissolved agar (55°C) and poured onto a culture dish of SCD-Trp-Ura or

5 SCG-Trp-Ura medium. Sterile filter discs are spotted with 5 μg or 15 μg of synthetic α-factor pheromone and placed onto the nascent lawn to induce growth arrest. The resulting zone of growth-arrested cells is documented after 2 days at 30°C.

Yeast expressing the Sec-1 G protein gamma subunit fusion and wild-type (non-mutated) syntaxin do not respond to α-factor induced growth arrest as exhibited by the

10 absence of a zone of inhibition around filter paper disks containing 5 or 15 μg of α-factor. This is contrast to cells which express the Sec-1 G protein gamma subunit fusion and syntaxin which contains a mutation that disrupts syntaxin-Sec-1 binding. In these cells, upon pheromone addition, the G protein subunits dissociate and G beta/gamma is free to activate a signaling pathway leading to growth arrest.

15 It should be understood that the foregoing discussion and examples merely present a detailed description of certain preferred embodiments. It therefore should be apparent to those of ordinary skill in the art that various modifications and equivalents can be made without departing from the spirit and scope of the invention. Once such variation includes the use of other signaling cascades which transmit signal through

20 membrane proteins into the cell. All references, articles and patents identified above are herein incorporated by reference in their entirety. This application is related to U.S. Provisional Application 60/082,940, filed April 24, 1998, which is herein incorporated by reference in its entirety.

Claims

What is claimed is:

1.   A method of identifying an agent that modulates at least one interaction between two proteins, comprising the steps of:

5   a)   exposing a cell to the agent, the cell comprising:

                       -a first protein fused to a G protein gamma subunit; and

                       -a second protein; and

              b)   determining whether the interaction of the G protein gamma subunit with an effector molecule has been modulated, thereby identifying an agent that modulates at

10   least one interaction between the two proteins.

2.   The method of claim 1, wherein the cells are provided in an aqueous solution and the exposure is carried out by adding the agent to the aqueous solution.

3.   The method of claim 1, wherein said cell does not comprise an endogenous G protein gamma subunit capable of interacting with the effector molecule.

15   4.   The method of claim 1, wherein the cell expresses an active endogenous effector molecule.

5.   The method of claim 1, wherein the second protein is a membrane protein.

6.   The method of claim 5, wherein the agent interacts with an extracellular domain of the second protein.

20   7.   The method of claim 1, wherein the effector molecule is selected from the group consisting of kinases, arrestins, nucleotide cyclases, phosphodiesterases, phospolipases, small molecule transporters and channels.

8.   The method of claim 1, wherein the second protein is expressed in its native form.

9.   The method of claim 1, wherein the agent inhibits an interaction between the first protein and the second protein.

5   10.   The method of claim 9, wherein the interaction of the G protein gamma subunit with the effector molecule is enhanced upon addition of the agent.

11.   The method of claim 1, wherein the agent promotes an interaction between the first and second proteins.

12.   The method of claim 11, wherein the G protein gamma subunit is sequestered
10   from the effector molecule upon addition of the agent.

13.   The method of claim 1, wherein the interaction of the G protein gamma subunit with an effector molecule confers a detectable phenotype upon the host cell.

14.   A method for detecting one or more interactions between two proteins, comprising the steps of:

15   a)   providing a cell with a first protein fused to a G protein gamma subunit and a second protein; and

          b)   determining whether the interaction of the G protein gamma subunit with an effector molecule has been modulated, thereby determining whether the first and second proteins interact.

20   15.   The method of claim 14, wherein said cell does not comprise an endogenous G protein gamma subunit capable of interacting with the effector molecule.

16.   The method of claim 14, wherein the cell does express an active endogenous effector molecule.

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17.   The method of claim 14, wherein the first protein is an unknown member of a library of proteins fused to a G protein gamma subunit.

18.   The method of claim 14, wherein the second protein is a membrane protein.

19.   The method of claim 14, wherein the effector molecule is selected from the group

5   consisting of kinases, arrestins, nucleotide cyclases, phosphodiesterases, phospolipases, small molecule transporters and channels.

20.   The method of claim 14, wherein the second protein is expressed in its native form.

21.   The method of claim 14, wherein the interaction of the G protein gamma subunit

10   with an effector molecule confers a detectable phenotype upon the host cell.

22.   The method of claim 14, wherein the second protein is an unknown protein encoded by a cDNA library.

23.   A nucleic acid molecule which encodes a protein fused to a G protein gamma subunit.

15   24.   A vector comprising the nucleic acid molecule of claim 23.

25.   A host cell comprising the vector of claim 24.

26.   A kit for detecting protein-protein interactions, comprising a nucleic acid molecule of claim 23.

27.   A method for identifying one or more interactions between a first and second

20   protein, comprising the steps of:

a)   transforming or transfecting a host cell population with a vector which expresses a first protein fused to a G protein gamma subunit;

b)   transforming the host cell population with a vector which expresses a second protein:

5   c)   culturing the cells under conditions which express the first and second proteins; and

d)   determining the activity of an effector molecule which is capable of interacting with the G protein gamma subunit, thereby identifying one or more interactions between the first and second proteins.

10   28.   The method of claim 27, wherein the G protein gamma subunit is sequestered from the effector molecule upon interaction of the first and second proteins.

29.   The method of claim 27, wherein step (a) comprises transforming or transfecting said host cell population with a plurality of vector molecules comprising a library of cDNA molecules fused in frame to a gene encoding a G protein gamma subunit.

15   30.   The method of claim 27, wherein step (b) comprises transforming or transfecting said host cell population with a plurality of vector molecules comprising a library of cDNA molecules.

31.   The method of any one of claims 27-30, wherein the first and second proteins are encoded by the same vector.

20   32.   A method of identifying binding partners of a protein, comprising the steps of:

a)   transforming or transfecting host cells with a library comprising a population of nucleic acid molecules which express a first protein fused to a G protein gamma subunit to produce a host cell population, said population of nucleic acids differing with respect to the first protein fused to a G protein gamma subunit;

25   b)   transforming the host cell population with a vector which expresses a second protein:

c)   culturing said host cell under conditions which express said first and
second protein; and

d)   determining the activity of an effector molecule which is capable of
interacting with the G protein gamma subunit.

5   33.   A method of identifying binding partners of a protein, comprising the steps of:

a)   transforming or transfecting a host cell with a nucleic acid molecule
which express a first protein fused to a G protein gamma subunit;

b)   transforming the host cell with a vector which expresses a second protein;

c)   culturing said host cell under conditions which express said first and
10   second protein; and

d)   determining the activity of an effector molecule which is capable of
interacting with the G protein gamma subunit.

34.   The method of claim 33, wherein the second protein is an unknown protein
encoded by a cDNA library.

15   35.   A method for identifying mutations in a protein which effect one or more
interactions between two proteins, comprising the steps of:

a)   providing a cell with a first protein fused to a G protein gamma subunit
and a second protein presumed to contain the mutation; and

b)   determining whether the interaction of the G protein gamma subunit with
20   an effector molecule has been modulated, thereby determining whether the interactions
of the first and second proteins is effected by the mutation.

36.   A method of identifying mutations in a protein which affect one or more
interactions between two proteins, comprising the steps of:

a)   transforming or transfecting a host cell with a nucleic acid molecule
25   which express a first protein fused to a G protein gamma subunit;

b)   transforming the host cell with a vector which expresses a second protein;

c)   culturing said host cell under conditions which express said first and
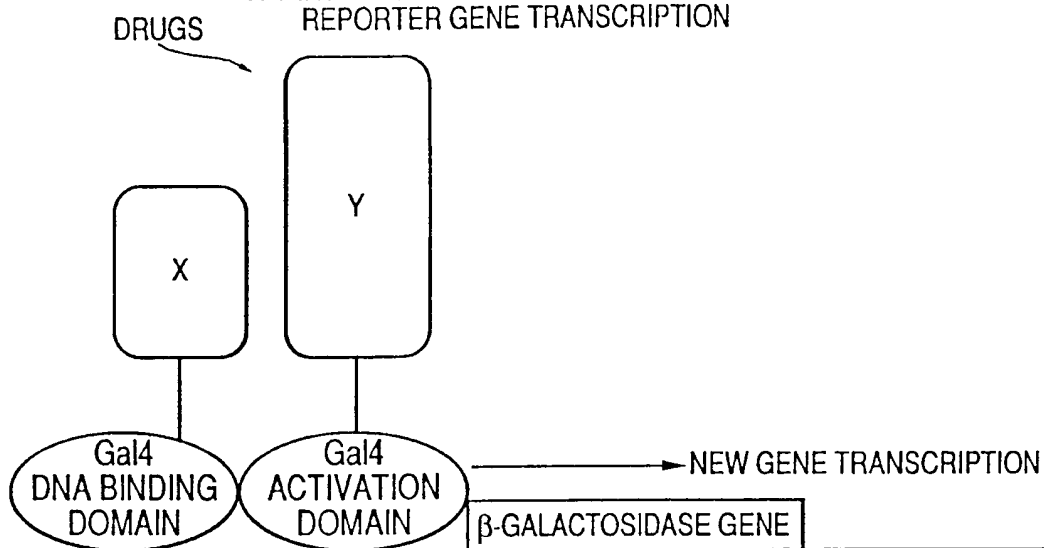
second protein; and

d)   determining the activity of an effector molecule which is capable of

interacting with the G protein gamma subunit.

5   37.   The method of claim 34, wherein either the first or second proteins

contain a mutation.

FIG. 1A

TRADITIONAL 2-HYBRID METHOD
X-Y INTERACTION IN THE NUCLEUS DRIVES
REPORTER GENE TRANSCRIPTION

DRUGS

Y

X

Gal4
DNA BINDING
DOMAIN

Gal4
ACTIVATION
DOMAIN

→ NEW GENE TRANSCRIPTION

β-GALACTOSIDASE GENE

FIG. 1B

NEW METHOD
X-Y INTERACTION AT THE MEMBRANE BLOCKS Gβ/γ—MEDIATED SIGNAL
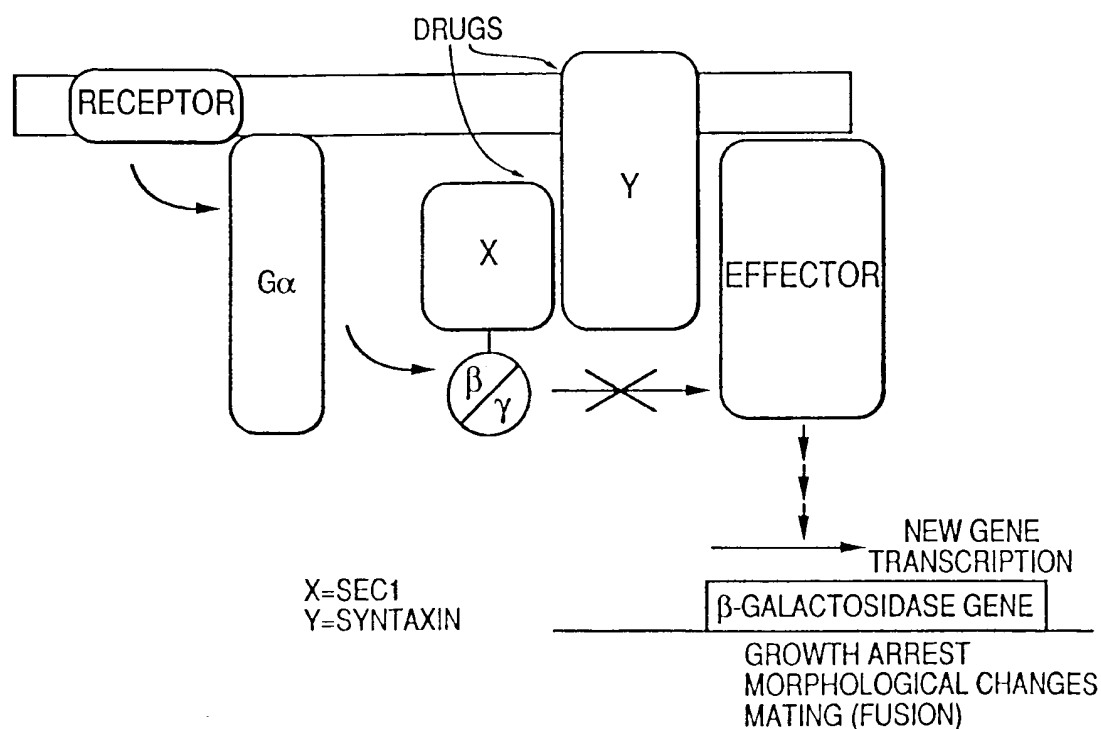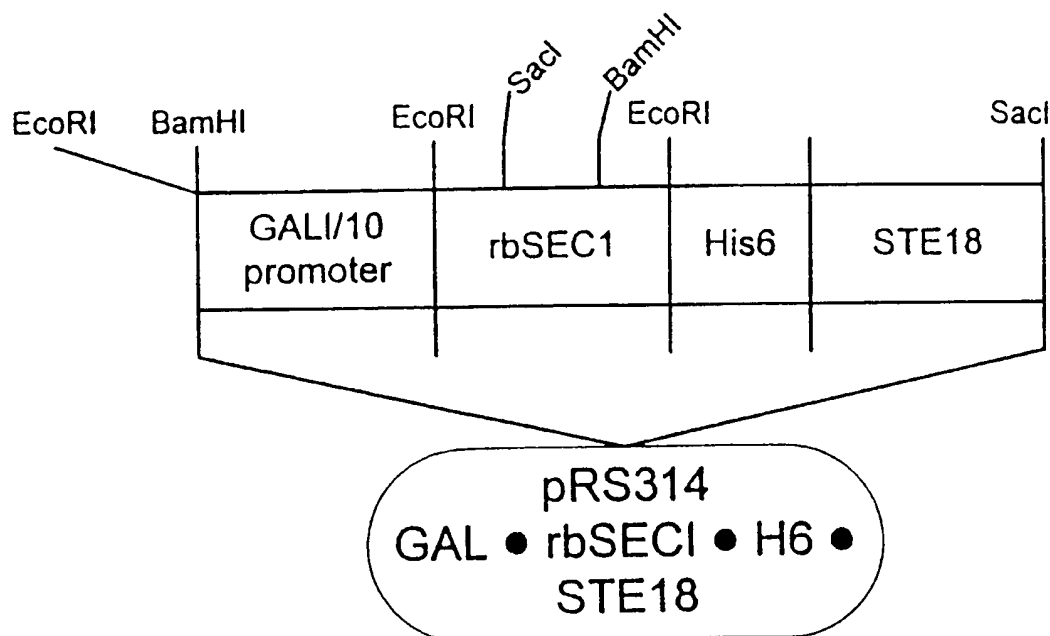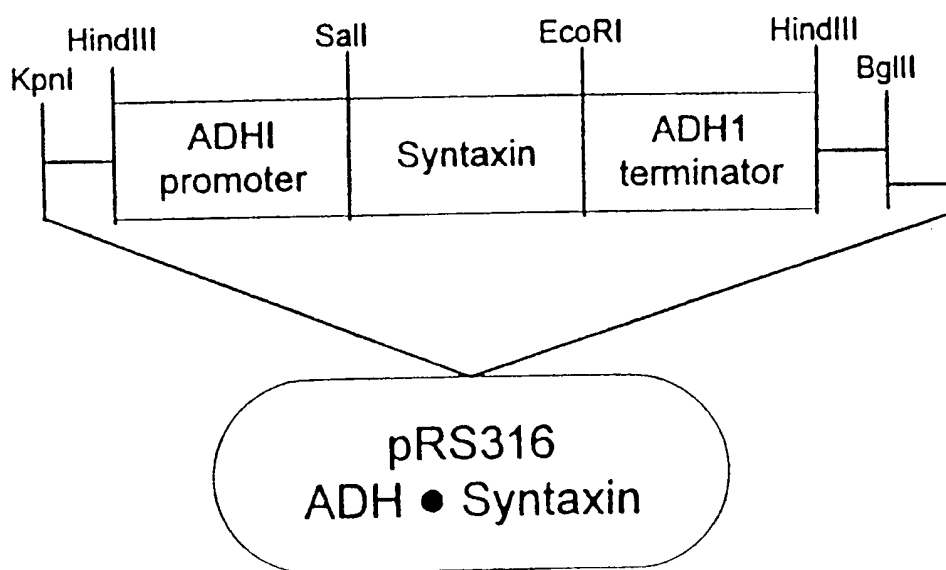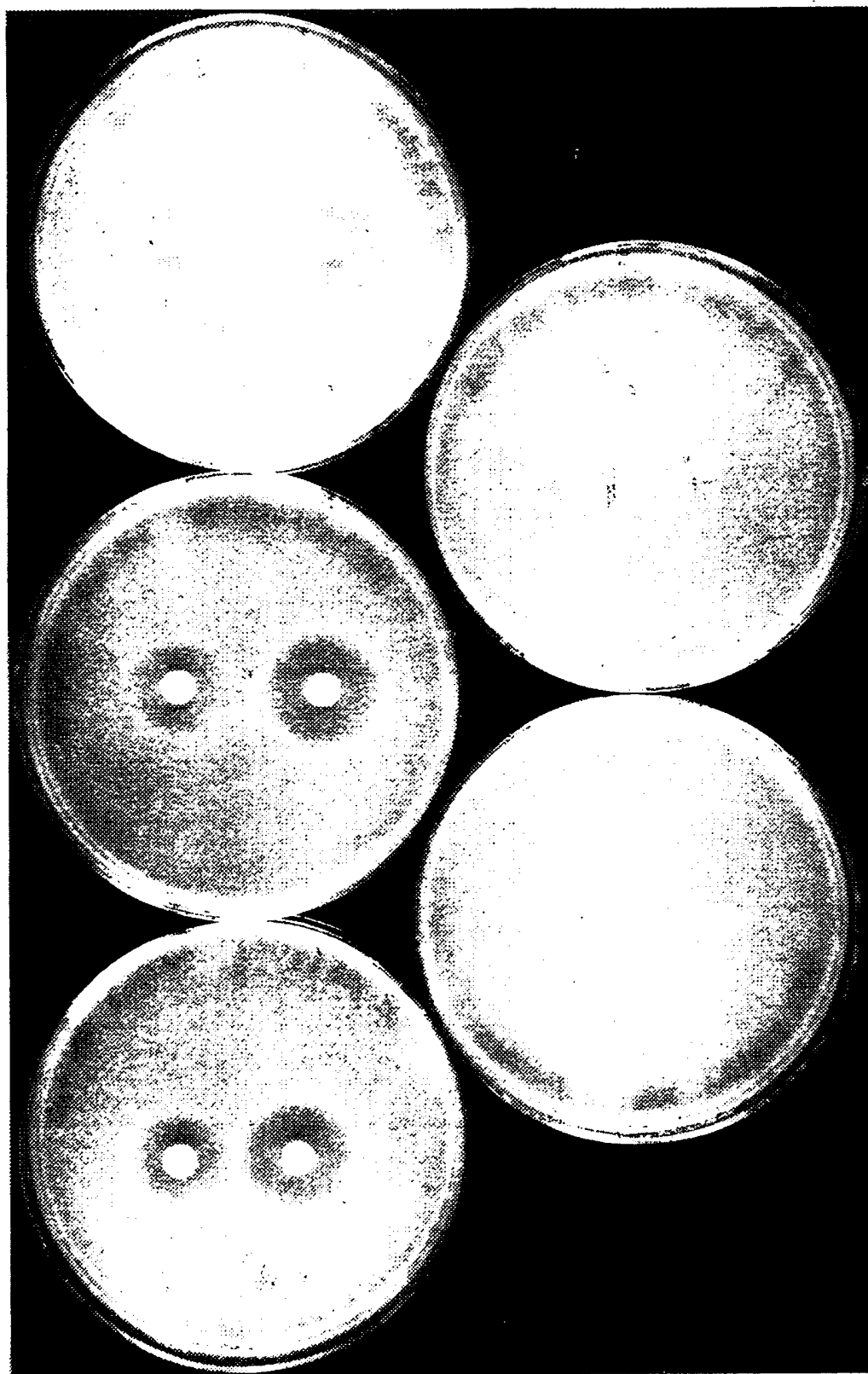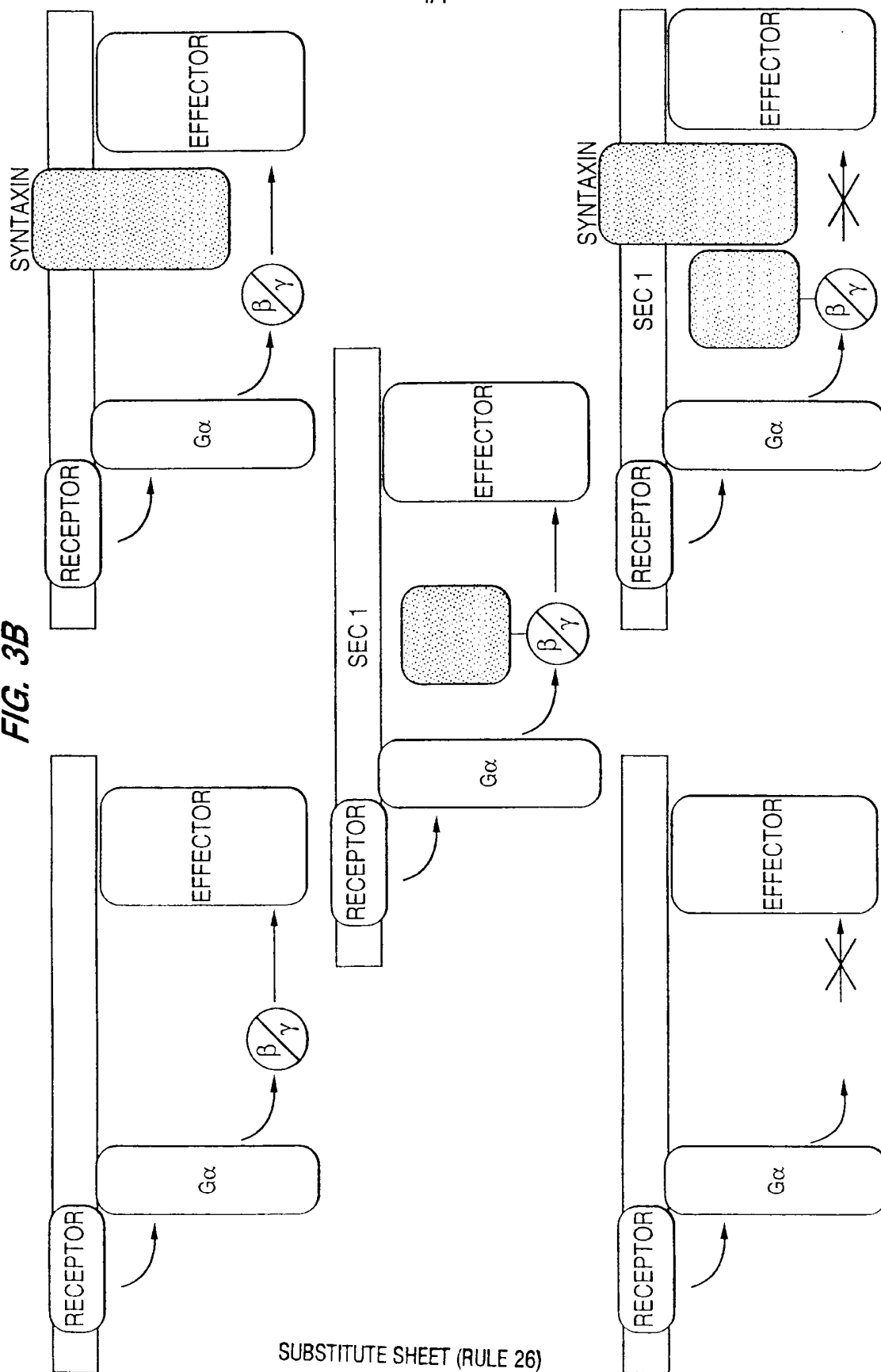
DRUGS

RECEPTOR

Y

Gα

X

EFFECTOR

β/γ

NEW GENE
TRANSCRIPTION

β-GALACTOSIDASE GENE

X=SEC1
Y=SYNTAXIN

GROWTH ARREST
MORPHOLOGICAL CHANGES
MATING (FUSION)

FIG. 2A

FIG. 2B

FIG. 3A

FIG. 3B

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A61K 38/00; G01N 33/53; C12P 21/06

US CL   : 435/7.1, 69.1, 69.7, 69.7; 530/330

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/7.1, 69.1, 69.7, 69.7; 530/330

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, SCISEARCH, EMBASE, BIOSIS, WPIDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,739,029 A (KING et al) 14 April 1998, see the entire document.	1-22
Y	US 5,482,835 A (KING et al) 09 January 1996, see the entire document.	1-22

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 JULY 1999	**20 AUG 1999**
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer P. PONNALURI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/08912

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-22

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single
inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees
must be paid.

Group I, claims 1-22, drawn to a method of identifying an agent that modulates at least one interaction between two
proteins and a method for detecting one or more interactions between two proteins using a cell with a first protein fused
to a gamma subunit.
Group II, claims 23-26, drawn to a nucleic acid molecule which encodes a protein fused to a G protein gamma subunit,
a vector and a host cell comprising nucleic acid of claim 23.
Group III, claims 27-34, drawn to a method for identifying one or more interactions between a first and second protein
and a method of identifying binding partners of a protein.
Group IV, claims 35-37, drawn to a method of identifying mutations in a protein which effect one or more interactions
between proteins.

The inventions listed as Groups I, II, III and IV do not relate to a single inventive concept under PCT Rule 13.1
because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The special technical feature of group I inventions is a method of identifying an agent using a cell comprising a first
protein fused to a G protein gamma subunit and a second protein; the special technical feature of group II inventions is a
nucleic acid molecule which encodes a protein fused to a gamma subunit; the special technical feature of group III
inventions is a method for identifying one or more interactions between a first and a second protein by transforming or
transfecting a host cell population with a vector or a with a library comprising nucleic acid molecule which express a
first protein fused to a G protein gamma subunit; the special technical feature of group IV inventions is a method of
identifying mutations in a protein using a cell with a first protein fused to a G protein gamma subunit and a second
protein presumed to contain mutations. Since the special technical feature of group I inventions are not present in groups
II-IV, unity of invention is lacking.